(72) Inventors: GOODMAN, Howard, M. ; 10 The Ledges Road, Newton Centre, MA 02159 (US). DONN, Gunter ; Sachsenring 35, D-6238 Hofheim (DE).

INF C12 N9/00

INF C12 N3/26

INF C12 N15/26

(54) Title: PLANT CELLS RESISTANT TO HERBICIDAL GLUTAMINE SYNTHETASE INHIBITORS
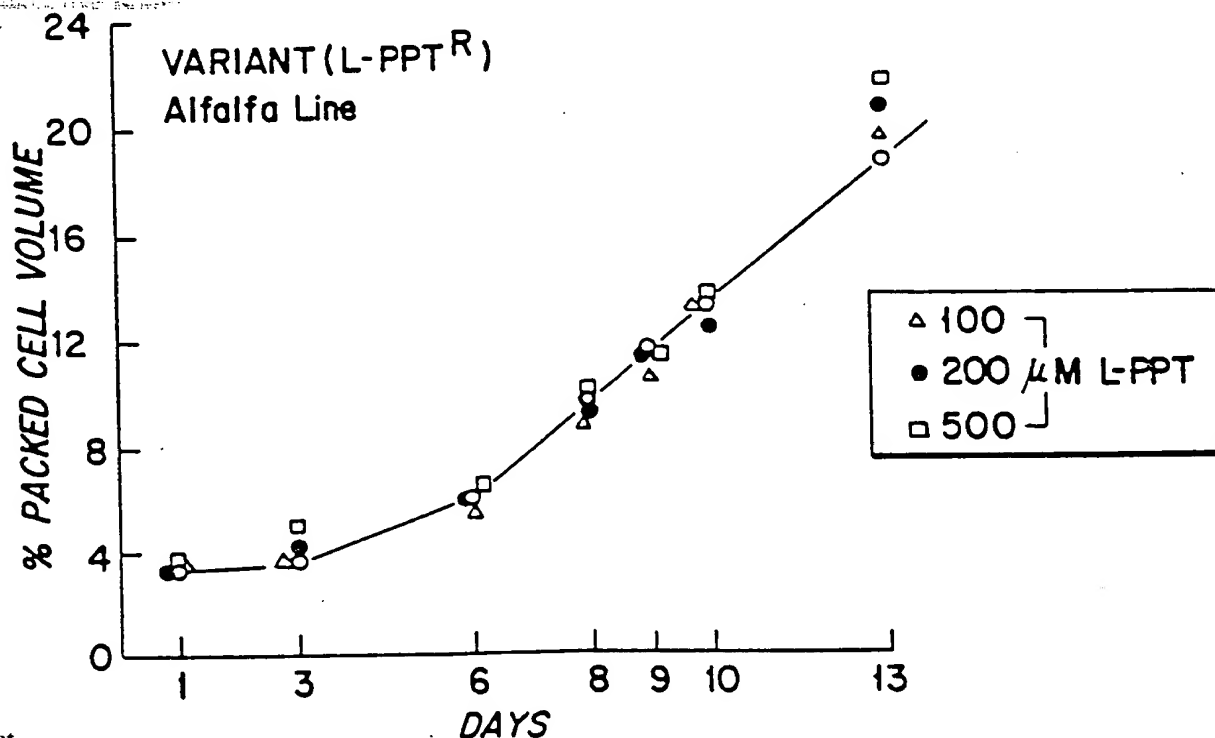
(57) Abstract

A plant cell which is resistant to a herbicidal glutamine synthetase inhibitor, wherein the resistance is caused by levels of GS activity which, when present in an otherwise herbicidal GS inhibitor sensitive plant cell, render the cell substantially resistant to the herbicidal GS-inhibitor.

-1-

DESCRIPTION:

PLANT CELLS RESISTANT TO HERBICIDAL GLUTAMINE SYNTHETASE INHIBITORS

TECHNICAL FIELD

The present invention relates to the use of recombinant DNA technology for the transformation of plant cells and, more specifically, the design and construction of plant cells which are resistant to herbicidal plant glutamine synthetase inhibitors, such as phosphinothricin.

BACKGROUND ART

Glutamine Synthetase (GS) is a plant enzyme which has a central role in the assimilation of ammonia and in the regulation of nitrogen metabolism. Since in most plants glutamine synthetase is, via the glutamine synthethase/glutamate synthase pathway (Fig. 1), the only efficient way to detoxify ammonia released by nitrate reduction, amino acid degradation or photo-respiration, plants are very susceptible to potent inhibitors of glutamine synthetase.

-2-

One of the most potent glutamine synthetase inhibitors known at present is phosphinothricin (1) (hereinafter PPT):

$$\begin{array}{c} O \\ \parallel \\ CH_3-P-OH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CH-NH_2 \\ | \\ COOH \end{array} \qquad (1)$$
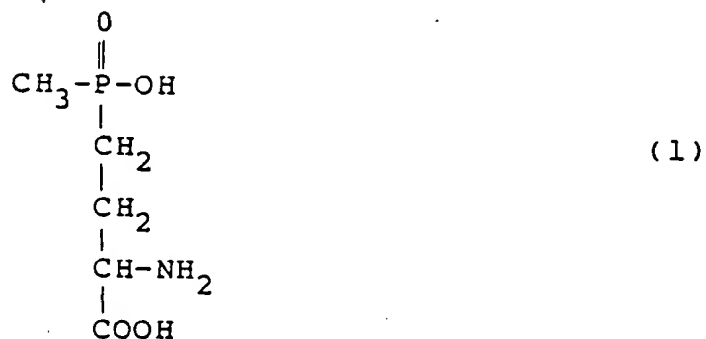
PPT is a glutamic acid analogue. The compound was initially isolated from a tripeptide antibiotic produced by Streptomyces viridochromogenes (Bayer, E. et al., Helvetica Chimica Acta, 55: 224 (1972), see also German Patent DOS 2717440, Hoechst, A.G.). PPT is a potent competitive inhibitor of glutamine synthetase from E. coli with a K_i of 0.0059 mM.

Schwerdtle, F. (Zeitschrift fur Pflanzen-Krankheiten und Pflanzenschutz, IX, 431-440 (1981)) demonstrated that PPT is a non-selective foliar herbicide for the control of undesirable mono and dicotyledonous plants in orchards, vineyards, plantations with minimum tillage, direct drilling, and as a harvest aid. Field trials in West Germany, Spain, South Africa, U.S.A. and Japan showed that most dicotyledonous weeds were well controlled. For monocotyledonous weeds somewhat higher quantities were needed for good control. Leason, M. et al., (Phytochemistry, Volume 21: 855-857 (1982)) demonstrated that PPT is a mixed competitive inhibitor of pea leaf glutamine synthetase with an apparent K_i value of 0.073 mM.

-3-

It would be of great interest to be able to confer resistance to PPT, as well as to other GS inhibitors to selected plants, since herbicidal selectivity is quite crucial in any commercially useful herbicide. PPT, as indicated, is non-selective.

There is some precedent for the existence of glutamine synthetases resistant to other compounds. It is known that methionine sulfoximine (MSO), another glutamate analogue, is a mixed competitive inhibitor (K_i value of 0.16mM) of pea leaf glutamine synthetase (Leason, M., et al., Phytochemistry 21:855-857 (1982)). Miller, E. S. and Brenchley, J. E. (The Journal of Biological Chemistry 256:11307-11321 (1981)) studied the properties of several mutants of Salmonella resistant to MSO. One mutation apparently altered glutamine synthetase at the ammonia binding domain, conferring MSO resistance. More recently, Young and Ringold (ibid 258: 11260-11266 (1983)) have reported that mouse 3T6 cells grown in the presence of MSO developed resistance thereto. MSO resistant cells had mRNA enriched for glutamine synthetase, and the authors suggested that this observation implied an amplification of the gene. See also Sanders and Wilson, EMBO. J., 3:65-71 (1984). Neither the Miller, Young, nor Sanders studies were reported on plant GS.

Further, prior to the present invention no studies have been reported on attempts to confer resistance to herbicidal GS inhibitors, such as PPT, by manipulating the glutamine synthetase genes in plant cells.

-4-

It would therefore be desirable to develop plant cells which are resistant to herbicidal inhibitors of GS, such as PPT, by manipulating the plant glutamine synthetase genes in said cells. In such manner, it would be possible to confer herbicidal selectivity to any given plant.

DISCLOSURE OF THE INVENTION

The present invention arose out of the discovery that resistance to PPT in plants can arise due to overproduction of glutamine synthetase, a phenomenon which, in the initial experiments, was shown to be due to an underlying gene amplification mechanism. Upon applying selective pressure on certain plant cells in tissue culture, it was possible to isolate PPT resistant strains. The resistance, however, was not due to the presence of a structural mutant of glutamine synthetase which had less affinity for PPT, but rather, due to gene amplification and concommitant increased concentrations of the enzyme in the plant cells. Out of these initial observations arose the concept of the invention of developing plant cells which overproduce glutamine synthetase (and thus show herbicidal GS-inhibitor resistance), either by gene amplification or by other, different, mechanisms than gene amplification.

The invention is therefore based on producing a plant cell which is resistant to a herbicidal glutamine synthetase (GS) inhibitor, wherein said resistance is caused by plant cell levels of GS activity which, when present in an otherwise herbicidal

-5-

GS-inhibitor sensitive plant cell, render said cell substantially resistant to said herbicidal GS inhibitor.

The invention can be accomplished by a variety of methods and thus encompasses various embodiments. For example, in one embodiment, the invention is based on producing a plant cell carrying a gene combination comprising:

A)   a first genetic sequence coding for a glutamine synthetase (GS) functional in said plant cell, operably linked to

B)   a second genetic sequence capable of increasing the levels of expression of said first genetic sequence such that when said combination is present in an otherwise herbicidal GS-inhibitor sensitive plant cell, said cell is substantially resistant to said herbicidal GS-inhibitor.

The invention also comprises a gene combination as described, present in the genome or in a replicating extrachromosomal element of a plant species, which species is heterologous for said first or second genetic sequence or for both.

In another embodiment, the invention is based on producing a herbicidal GS-inhibitor resistant plant cell which contains significantly increased levels of GS activity by virtue of having a significantly larger number of copies of the wild GS gene than the corresponding herbicidal GS-inhibitor sensitive plant cell. In this embodiment, a plant can be obtained by inducing GS gene amplification by selective pressure in cell culture to obtain a resistant variant or strain,

-6-

followed by growth and development thereof into a callus and full grown plant, and sexual reproduction thereof. Alternatively, a plant can be obtained by inducing GS gene amplification by selection in cell culture of a resistant donor variant or strain, followed by protoplast fusion of the donor cells with an appropriate acceptor cell to yield a morphogenetic hybrid capable of further growth and development.

As another alternative, a resistant plant cell which contains a significantly larger number of copies of the wild gene than the corresponding sensitive cell can be obtained by introducing multiple expressible copies of the wild GS gene into an appropriate extra-chromosomal element capable of replication, or into the genome of the plant cell itself. This can be accomplished by transformation at the cell culture stage or at the whole plant stage. Stable multicopy organelles carrying the GS wild gene can also be used to introduce significantly larger numbers of the GS gene into a cell.

The invention further comprises herbicidal GS inhibitor-resistant, transformed plant cells per se, which in their otherwise untransformed state would be herbicidal GS inhibitor sensitive. Whole plants which are herbicidal GS inhibitor resistant are also included.

The invention also comprises intermediate vehicles capable of serving as transformation vectors for plant cells carrying genetic information as described, capable of conferring herbicidal GS inhibitor-resistance, and methods of conferring resistance to plant cells.

The invention further comprises a method of plant control achieved by contacting herbicidal GS inhibitor sensitive plants with plant controlling amounts of a herbicidal GS inhibitor, while in the presence of, and simultaneously contacting plants made resistant to said inhibitor by the methods described.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be better understood by reference to the accompanying Figures where

Figure 1 shows the glutamine synthetase/glutamate synthase cycle, wherein it is shown that GS catalyzes the formation of glutamine from glutamic acid and ammonia in a reaction driven by the hydrolysis of ATP to ADP and inorganic phosphate. The amide nitrogen of glutamine provides the source of nitrogen for many biosynthetic reactions, indicating a central role for GS in nitrogen metabolism. The herbicidal GS inhibitor, by inhibiting GS, prevents the biosynthesis of glutamine, thereby preventing ammonia detoxification.

Figure 2 shows the growth characteristics of a wild type (PPT-sensitive) alfalfa cell line in the absence (o) and in the presence of 25 (△), 50 (●) and 100 (□) um L-PPT.

Figure 3 shows the growth characteristics of a variant PPT resistant alfalfa cell line in the absence (o) and in the presence of 100 (△), 200 (●) and 500 (□) um L-PPT.

-8-

BEST MODE OF CARRYING OUT THE INVENTION

In the description that follows, a number of terms used in recombinant DNA, plant genetics technology and in the present invention are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided:

Nucleotide. A monomeric unit of DNA or RNA consisting of a sugar moiety, a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose). The combination of a base and a sugar is called a nucleoside. Each nucleotide is characterized by its base. The four DNA bases are adenine (A), guanine (G), cytosine (C) and thymine (T). The four RNA bases are A, G, C and uracil (U).

Genetic sequence. A linear array of nucleotides connected one to the other by phosphodiester bonds between the 3' and 5' carbons of adjacent pentoses.

Functional Genetic Sequence. A genetic sequence coding for a polypeptide having desired activity, regardless of whether the sequence is shorter or longer than that of the full length sequence for the polypeptide. It is also referred to as "functional gene."

Codon or Triplet. A DNA sequence of three nucleotides which encodes through mRNA an amino acid, a translation start signal or a translation termination signal. For example, Codons TTA, TTG, CTT, CTC, CTA and CTG encode for the amino acid leucine. TAG, TAA,

and TGA are translation stop signals, and ATG is a translation start signal.

Reading frame. The grouping of codons during translation of mRNA into an amino acid sequence. During translation, the proper reading frame must be maintained. For example, the sequence GCTGGTTGTAAG may be translated into three reading frames or phrases depending on whether one starts with G, with C, or with T, and thus may yield three different peptide products.

Two sequences are in operable linkage when the reading frame of one sequence is linked to the other so that both operate in combination as if they would be present independently.

Transcription. The process of producing mRNA from a functional gene.

Translation. The process of producing a polypeptide from mRNA.

Expression. The process, starting with a functional gene, to produce its polypeptide, being a combination of transcription and translation.

Cloning vehicle. A plasmid, phage DNA, or other DNA sequences which are able to replicate in a host cell, which are characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the DNA, and which contain a marker suitable for use in the identification of transformed cells. A typical example is an antibiotic resistance marker. The word "vector" is sometimes used for a cloning vehicle.

Expression vehicle.   A vehicle, analogous to a cloning vehicle, which is particularly useful for production in host cells of a polypeptide by expression of the functional gene coding for said polypeptide, present in the vehicle.

Replication vehicle.   A cloning or expression vehicle.

Phage or bacteriophage.   A bacterial virus which may consist of DNA sequences encapsulated in a protein envelope or coat.

Plasmid.   A non-chromosomal double stranded DNA sequence comprising an intact "replicon," such that the plasmid is replicated in or incorporated into the genome of a host cell.   When the plasmid is placed within a unicellular or multicellular organism, the characteristics of that organism may be changed or transformed as a result of the DNA of the plasmid. For example, a plasmid carrying the gene for kanamycin resistance transforms a cell previously sensitive to kanamycin into one which is resistant to it.

Cloning.   The process of obtaining a population of organisms or DNA sequences derived from one such organism or sequence by asexual reproduction.

Expression control sequence.   A genetic sequence that controls and regulates expression of functional genetic sequences when operably linked to those sequences.   They include sequences known to control the regions which regulate that expression.   They comprise both promoter and terminator sequences.

Plant Promoter.   An expression control sequence which is capable of causing the expression in said

-11-

plant of any homologous or heterologous genetic sequences or sequences operably linked to such promoter.

Overproducing Plant Promoter (OPP). A plant promoter capable of causing the expression in a transformed plant cell of any operably linked functional genetic sequence or sequences to levels (measured by mRNA or polypeptide quantities) which are substantially higher than the levels naturally observable in host cells not transformed with said OPP.

Degeneracy. An informational property according to which each amino acid in nature can be coded by more than one codon. For example, leucine can be coded by TTG, TTA, CTA, CTT, CTC or CTG.

By degenerate variations as used in the present application and claims, is meant any variation of the polynucleotide fragments of the invention due to the degeneracy of the genetic code. For example, as long as the resulting material still codes for functional GS, or portions thereof, such material is designed to be included in the present invention.

Glutamine Synthetase (GS). The definition of this enzyme is functional, and includes any glutamine synthetase capable of functioning in a given desired plant to transform glutamic acid to glutamine in the GS cycle. The term therefore includes not only the enzyme from the specific plant species involved in the genetic transformation, but may include GS from other plant species or microbes or even other eukaryotes, if such GS is capable of functioning in the transformed

-12-

plant cells. The terms include proteins or polypeptides having more or less than the total structural length of natural plant GS, such as functional partial fragments of GS, or their analogues.

Phosphinothricin (PPT). The compound of formula (1), supra, in its biologically active form. It may be the L-, or D- or D,L- forms, and may be alone or in combination with other inert or active compounds which do not interfere with PPT activity.

The invention comprises at its most fundamental level a plant cell which is resistant to a herbicidal glutamine synthetase inhibitor wherein the resistance is caused by levels of GS activity which, when present in an otherwise herbicidal GS inhibitor-sensitive plant cell, render the cell substantially resistant to the herbicidal GS inhibitor.

The terms "herbicidal glutamine sythetase inhibitor" are meant to include any inhibitor, competitive or noncompetitive, that significantly decreases the glutamine synthetase activity of a plant cell of a given species and, as a consequence thereof, causes herbicidal effects in the plant cell. The herbicide resistant plant cell or whole plant survives without irreversible damage a herbicidal GS inhibitor concentration which is lethal for wild type individuals of the same species. Normally, a five-fold or higher increase in herbicidal GS inhibitor resistance is considered significant. However, this number varies from plant species to plant species, and from herbicide to herbicide. It is therefore not feasible to provide a

-13-

single level for all of the several plant species and herbicides covered by the present invention. Such level, however, can be readily ascertained by those skilled in the art.

Among the herbicidal GS inhibitors covered by the invention are phosphinotricin, methionine sulfoximine, as well as other glutamic acid analogues.

The glutamine synthetase may or may not be from the specific plant cell being transformed. All that is necessary is that the genetic sequence for the enzyme be expressed, and produce, a functional enzyme in the final plant cell. Thus, the invention comprises plant cells containing either homologous GS genes or heterogolous GS genes (and their respective expression products). Broadly, the enzyme might also be that of other plant species, or even enzymes from different organisms, such as microorganisms or animals. Preferred are plant glutamine synthetase genes and their expression products. Of particular interest are glutamine synthetase genes from the particular plant species which serves as the host in the genetic manipulation, i.e., homologous GS genes. One such glutamine synthetase gene utilizable in the rDNA molecules of the invention is illustrated in Example 2.

Any plant cell which is sensitive to herbicidal GS inhibitors, which is capable of undergoing genetic manipulation by the genetic constructs or methods of the invention, and is capable of expressing said constructs, can be used in the present invention. Among dicotyledonous plants are included various species of potato (Solanum tuberosum); tomato (Lycopersicon esculentum); pepper (Capsicum annumm); tobacco (Nicotiana

tabacum); various species of Brassica, especially rapeseed (Brassica napus), various legumes, for example alfalfa (Medicago sativa), clover (Trifolium spec.), soybean (Glycine max), groundnut (Arachis hypogaea), various species of beans (Phaseolus spec., Vicia spec., Vigna spec.), peas (Pisum sativum), root crops as beets (Beta vulgaris), carrots (Daucus carota) and sweet potatoes (Ipomoea batatus).

There are a variety of embodiments encompassed in the broad concept of the invention. The herbicidal GS-inhibitor resistant plant cell can contain significantly higher levels of GS activity by any of a variety of mechanisms and/or genetic constructs.

For example, the invention comprises, in one of its embodiments, a combination of two genetic sequences: (a) a first genetic sequence coding for a glutamine synthetase functional in a given plant cell, operably linked downstream from (b) a second genetic sequence capable of increasing the levels of gene product of said first sequence such that when the combination is present in an otherwise herbicidal GS-inhibitor sensitive plant cell, said cell is substantially resistant to said herbicidal GS-inhibitor.

The second genetic sequence may be a promoter or an enhancer sequence. If a promoter, it may be a GS promoter or a promoter of another structural gene. In either of the latter two events, the promoter useful in the combination is an overproducing plant promoter. The only essential characteristic of such promoter is

that, when in operable linkage with the genetic
sequence for glutamine synthetase, it be capable of
promoting expression of said glutamine synthetase to
levels such that when the combination is present in an
otherwise herbicidal GS-inhibitor sensitive plant
cell, the cell is substantially resistant to said
GS-inhibitor.. Thus, the choice of what plant promoter
to use is ruled by functional considerations. The
plant promoter will be heterologous or homologous to
the host cell. The native, endogenous promoter of
said host cell would be incapable of causing resist-
ance by overproduction of GS, since such native
promoter normally promotes expression of GS to levels
which are so low as to be substantially or completely
inhibited by normally used plant-controlling herbicide
concentrations. Thus, under one embodiment of the
present invention, the native promoter is replaced by
an OPP.

Among useful OPP's are included the promoter of
the small subunit (ss) of the ribulose bi-phosphate
carboxylase, and of the chlorophyll a/b binding pro-
tein. The expression of these two genes has been
shown to be light induced at the transcriptional level
in green tissue (see, for example, Genetic Engineering
of Plants, An Agricultural Perspective, A. Cashmore,
Plenum, New York 1983, pages 29-38, Coruzzi G. et al.,
The Journal of Biological Chemistry, 258: 1399 (1983),
or Dunsmuir, P., et al., Journal of Molecular and
Applied Genetics, 2:285 (1983)).

The invention extends to any plant cell modified
according to the methods described, or modified by any

-16-

other methods which yield herbicidal GS-inhibitor resistance. The plant cell may be alive or not, by itself, in tissue culture or as part of a multicellular plant or a part thereof. Such a multicellular plant, which in its untransformed state is herbicidal GS-inhibitor sensitive, would be resistant when its cells are resistant according to the invention.

Parts obtained from the plant, such as flowers, seeds, leaves, branches, fruit and the like are also covered by the invention, as long as these parts comprise herbicidal GS-inhibitor resistant cells, as noted. In particular, plant parts may be alive or not. Thus, a genetically modified tomato, carrot or tobacco leaf obtained from a resistant tomato, carrot or tobacco plant is included in the invention, even if separate from the plant of origin.

PROCESS OF PRODUCTION AND INTERMEDIATES USED THEREIN

Various methodologies are applicable to carry out the different embodiments of the invention. For example, if the herbicidal GS-inhibitor resistance is brought about by providing significantly increased copies of a structural GS gene, (as opposed to the previously mentioned overproduction of the wild gene) available methodologies include selection for a cell which is resistant by virtue of GS gene amplification, or, alternatively, introduction of multiple copies of the GS gene into the genome or into a replicating extrachromosomal element.

Selection is carried out in an appropriate culture
medium by cultivating plant cells in the presence of
stepwise increases of the herbicidal GS-inhibitor in
the medium. After a given period of time has elapsed,
such as, for example, a few weeks to several months,
it is possible to select a cell population which is
several-fold more resistant to the herbicidal GS-
inhibitor than the original plant cells. For example,
when the cells are alfalfa and the inhibitor is PPT,
it has been possible to obtain a PPT-resistant alfalfa
cell line after one year of stepwise PPT increases,
which line was twenty-fold more resistant to PPT than
the original line. When the resistant line was sub-
cultured for several months in the absence of the
inhibitor, a slow decline in the percentage of resis-
tant cells was observed in plating experiments on
inhibitor-containing agar media, but after more than
six months it was still possible to reselect highly
resistant cell clones. This was never possible by
plating wild-type cells.

    Regeneration of calli and grown plant from tissue
culture cells is known to those skilled in the art,
and it varies from species to species. See, for exam-
ple, Sheperd, Scientific American, 1982, herein
incorporated by reference. Generally, a suspension of
protoplasts containing multiple copies of the GS gene
or containing the genetic sequence combination is
first provided. Embryo formation can then be induced
from the protoplast suspensions, to the stage of
ripening and germination as natural embryos. The cul-
ture media will generally contain various amino acids,

and hormones such as auxin and cytokinins. It is advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

For the genotypes of some species, such as tobacco, alfalfa, potato, tomato, petunias, soy beans, rapeseed, and some fruit trees and carrots, regeneration has been clearly demonstrated in the prior art. Such regeneration is well within the skill of the art if the appropriate genotype and history is chosen by screening. Generally, after about more than one year of subculturing, the efficiency of plant regeneration decreases from a given cell culture. Therefore, regeneration is normally most successful if it is initiated shortly after the provision of an appropriate resistant cell culture.

Alternatively, and especially useful in cell cultures which are older than several months or one year, the amplified GS gene can be carried into a regenerative system by crossing and/or fusion. See, for example, Cocking, et al., Nature, 293:265 (1981) herein incorporated by reference. In this method, a herbicidal GS-inhibitor resistant cell line is provided by selection, and a culture suspension of rapidly dividing cells is obtained by methods well known to those of skill in the art. Protoplasts are

isolated therefrom, and preferably irradiated with an amount of radiation and for a time sufficient to in-activate, if not completely irreversibly damage, the host cells. For example, x-ray radiation of 20 Krads can be used advantageously. After irradiating the donor protoplasts, the same are fused with acceptor protoplasts from appropriate susceptible cells. Vari-ous methods exist to obtain fusions, such as, for example, polyethylene glycol fusion, high calcium treatment, combinations of both, or even recently, electrofusion. After fusion has occurred, selective growth of the fusion product can be carried out since the fusion product should be herbicidal GS-inhibitor resistant. In particular, if a donor is used which is no longer morphogenetic (i.e., the history of the donor culture is such that several months or upwards of a year may have already elapsed since the original selection), then a simple selection in herbicidal GS-inhibitor containing media can be carried out.

Another method of introducing genetic material into plant cells is to infect a wounded leaf of the plant with transformed A. tumefaciens bacteria. Under appropriate growth conditions a ring of callii forms around the wound. The callii are then transferred to growth medium, allowed to form shoots, roots and develop further into plants. Alternatively, grafting onto whole plants can also be done.

An alternative method to introduce multiple copies of the GS gene into the plant cell is by self-ligating a copy of a functional GS (genomic or cDNA) structural gene, utilizing methods well known to those of skill

-20-

in recombinant DNA technology. The structural genes, each containing its individual promoter, are operably linked to each other by means of appropriate linkers, and 10-30 copies of each gene can in certain instances be introduced into an appropriate replicable expression vehicle, such as a Ti plasmid. Alternatively, the genetic material can be micro-injected directly into plant embryo cells. In the case of monocotyledonous plants, pollen may be transformed with total DNA or an appropriate functional clone providing resistance, and the pollen then utilized to produce progeny by sexual reproduction.

Of course, any other methods utilizable to increase GS activity in a given cell to such levels as will make the cell herbicidal GS-inhibitor resistant can be utilized. Of particular interest in this invention is the operable linkage of a genetic sequence coding for a structural GS gene to another genetic sequence capable of overproduction of the gene product derived therefrom. This linkage of genetic sequences can be introduced into appropriate plant cells, for example, by means of the Ti plasmid.

The introduction of genetic material into plant cells, especially by use of the so-called tumor inducing (Ti) plasmid of Agrobacterium tumefaciens, is a reproducible and predictable technology (See, for example, Caplan, A. et al., "Introduction of Genetic Material into Plant Cells," Science: 815-821 (November, 1983); Schell, J. and Van Montagu, M., "The Ti Plasmid as Natural and as Practical Gene Vector for Plants," Bio/Technology: April 1983, pps. 175-180;

-21-

Horsch et al., "Inheritance of Functional Foreign
Genes in Plants," Science: 233, 496-498 (1984);
Fraley, R. T., et al., Proc. Nat. Acad. Sci. USA: 80,
4803 (1983), Watson, et al., Recombinant DNA. A Short
Course, Scientific American Books, 1983, pp. 164-173;
and Old and Primrose, Principles of Gene Manipulation,
2d Ed., U. Cal. Press, 1981, pp. 138-156, herein being
incorporated by reference).

Ti plasmids contain two regions essential for the
production of transformed cells. One of these, named
transfer DNA (T DNA), induces tumor formation. The
other, termed virulent region, is essential for the
formation but not maintenance of tumors. Transfer DNA,
which transfers to the plant genome, can be increased
in size by the insertion of the multiply linked GS
genes or of the gene combination of the invention
without its transferring ability being affected. By
removing the tumor causing genes so that they no
longer interfere, the modified Ti plasmid can then be
used as a vector for the transfer of the gene con-
structs of the invention into an appropriate plant
cell. The foreign DNA to be inserted is usually
introduced between the terminal sequences flanking the
T-region.

A particularly useful Ti plasmid vector is
pGV3850, a non-oncogenic derivative of the nopaline Ti
plasmid C58. (See Caplan, et al., supra) This vector
utilizes the natural transfer properties of the Ti
plasmid. The internal T-DNA genes that determine the
undifferentiated crown gall phenotype have been de-
leted and are replaced by any commonly used cloning

vehicle (such as pBR 322). The cloning vehicle se-
quence contained between T-DNA border regions serves
as a region of homology for recombination to reintro-
duce foreign DNA cloned in a derivative of the same
cloning vehicle. Any gene construct of the invention
cloned in such plasmid can thus be inserted into pGV
3850 by a single recombination of the homologous
sequences. Antibiotic resistance markers can be added
to the plasmid to select for the recombination event.
Herbicide resistance can of course be also used con-
comitantly or independently. The presence of the
nopaline synthase (nos) gene in this vector makes it
easy to monitor the efficiency of transformation using
pGV 3850. A callus in tissue culture can then be
tested for the presence of nopaline.

After transformation of the plant cell or plant,
the same may be selected by aid of an appropriate
marker, such as antibiotic resistance, or more rele-
vant, herbicide resistance, and then grown in conven-
tional ways. In tobacco, protoplast cultures three to
five days after the protoplast isolation are suitable
for the transformation by Agrobacteria harboring the
appropriate Ti-plasmid which contains in its T-DNA
region the hybrid GS-gene described. After two days
of cocultivation of the protoplast borne cells and the
Agrobacteria, the plant cells can be washed by cen-
trifugation and resuspended on fresh medium several
times. This removes most of the bacteria. The re-
maining bacteria are killed by a suitable antibiotic,
for example cefotaxime (400-1,000 ug/ml), added to the
protoplast culture medium. The cells are cultivated on

nonselective media until they form visible cell aggre-
gates (calli). Then they are plated on media contain-
ing the proper herbicide concentration which kills all
wild type cells. Only the transformants which express
the incorporated GS-hybrid gene survive and continue
to grow. These calli can be easily induced to regen-
erate shoots when transferred to Murashige and Shoog-
medium containing 1 mg/l 6-benzyladenine and 0.1 mg/l
naphthalene acetic acid. The shoots can be rooted on
hormone free MS-medium or directly on perlite or ver-
miculite, and transferred to pots. The plantlets are
ready to grow in the greenhouse and can be tested for
herbicide resistance.

Other systems, such as cauliflower mosaic virus,
CaMV (Hohn, B. et al., in "Molecular Biology of Plant
Tumors," Academic Press, New York 1982 pps. 549-560;
and Howell, United States Patent 4,407,956) can also
be used. The entire CaMV viral DNA genome is inserted
into a parent bacterial plasmid creating a recombinant
DNA molecule which can be propagated in bacteria.
After cloning, the recombinant plasmid is cleaved with
restriction enzymes either at random or unique sites
in the viral portion of the recombinant plasmid for
insertion of the gene combination of the invention. A
small oligonucleotide, described as a linker, having a
unique restriction site may also be inserted. The
modified recombinant plasmid again may be cloned and
further modified by introduction of larger pieces of a
gene construct into the unique restriction site of the
linker. The modified viral portion of the recombinant
plasmid is then excised from the parent bacterial

-24-

plasmid, and used to inoculate the plant cells or plants. This virus is described in the aforementioned Howell patent as being particularly good for insertion of genes capable of enhanced production of protein, greater tolerance to stress, resistance to pests and pesticides, nitrogen fixation, and the like.

Normally, the desired GS sequences are operably linked in vitro to each other or to an overproducing promoter, by known recombinant methodology. For example, the structural gene for GS, normally the genomic version thereof, is separated from its normal promoter by restriction on the region between such promoter and the initiation AUG codon. A transcriptional fusion with, e.g., the small subunit of the ribulose bi-phosphate carboxylase is then carried out. The construct is then inserted into an appropriate restriction site of the plant vehicle.

For example, when using CaMV DNA as a vehicle, the genetic construct is inserted into a site or sites in the viral DNA, without destroying infectivity of the viral DNA or its movement throughout the plant. Thus, the construct can be inserted into a variety of restriction sites, cloning the product and determining whether the essential characteristics of the virus have been retained. In this manner, one can rapidly isolate a relatively large amount of modified virus which can be screened for infectivity and movement. After the viral portion of the hybrid DNA plasmid has been modified, the modified virus may be excised from the hybrid DNA plasmid, and may be used to inoculate plants directly in linear form or ligated in circles.

-25-

Various techniques may be employed for infecting plant cells with CaMV vehicles. Young leaves may be mildly abraded and then contacted with the viral DNA. After infection, the viral DNA may be transmitted by aphids, where the aphid transmissible gene is operative. Mechanical techniques can also be employed. Alternatively, tissues or single cells may be infected.

USES

The use of vehicles containing the gene constructs of the invention is as intermediates in the preparation of whole herbicide-resistant plant cells and plants. Thus, not only the plant cells made resistant according to the methods of the invention, but also all of the vehicles or vectors, derive their utility from the utility of the final product, the whole plant.

The utility for a whole plant made herbicide resistant according to the invention is obvious. Such a plant, when brought into contact with otherwise plant controlling or suppressing amounts of herbicide, would be resistant thereto. This would allow herbicide treatment to be selective for any desired plant or group of plants.

In addition, the resistance to herbicide would enable its use as a selectable marker in the transfer of other genes to the plant, or cells thereof.

By the terms "plant controlling amounts of herbicidal GS-inhibitor" is meant to include functionally, an amount of herbicide which is capable of affecting the growth or development of a given plant. Thus, the

amount may be small enough to simply retard or sup-
press the growth or development, or the amount may be
large enough to irreversibly destroy the sensitive
plant.   Normally, most dicotyledonous plants and weeds
may be controlled at rates of between 0.5 to 1.5 kg/ha
ai.   For monocotyledonous plants, rates between 0.5
kg/ha ai, and up to about 2.0 kg/ha ai are normally
used.   The herbicide can, of course, be contacted with
the appropriate plant using well known spraying or
spreading methods.   For example, foliar administration
used in the prior art for control of weeds by PPT can
be used with PPT-resistant plants falling within the
invention.

The invention also encompasses a method of plant
control which comprises contacting a herbicidal
GS-inhibitor sensitive plant cell or plant, with plant
controlling amounts of herbicidal GS-inhibitor, where-
in the contact is carried out while the sensitive
plant cell or plant is present simultaneously with or
among the herbicidal GS-inhibitor resistant plant
cells or plants of the invention.   Thus, foliar her-
bicidal treatment of plants in a field or cultivar,
which plants include both those comprising herbicide
resistant plant cells and those comprising herbicide
sensitive plant cells, and wherein both are simulta-
neously contacted with the herbicidal GS inhibitor
during the treatment operation, is a method included
in the present invention.

Having now generally described this invention, the
same will become better understood by reference to
certain specific examples which are included herein
for purposes of illustration only and are not intended
to be limiting unless otherwise specified.

Example 1
Isolation and Characterization of PPT-Resistant Alfalfa Cell Culture.

An alfalfa cell line which is able to grow in the presence of 3 mM L-phosphinothricin in the culture medium was selected. Alfalfa was chosen since it is one of the model plants in which the regeneration of a whole plant from single isolated cells or protoplasts can be readily achieved.

The selection of the phosphinothricin resistant alfalfa line was done by stepwise increase of the inhibitor concentration in the liquid culture medium. Within 6 months a cell population was selected which was at least 20-fold more resistant to phosphinothricin than the original cell line. Measuring the packed cell volume of the suspension cultures revealed that wild type cells were completely inhibited by 2.5×10^{-5} M L-phosphinothricin. The resistant cells grew as well in the presence of 5×10^{-4} M of the compound as in its absence (Figures 2 and 3).

When the protein patterns of crude cell extracts of the susceptible and resistant cell lines were compared by SDS-polyacrylamide gel electrophoresis, the overproduction of a polypeptide in the molecular weight range 40-42 KD could be observed in the PPT-resistant cell line. Purified GS of alfalfa has the same molecular weight. The enzyme activity of the cell extracts was determined in parallel. The specific GS-activity in the resistant cells was 5-10 fold higher than in wild type cells. The specific activity

of GS in the PPT-resistant cell lines is in the same
order of magnitude as in root nodule tissue of alfal-
fa, which, as all nodule tissues of efficiently
dinitrogen fixing legumes, has high GS-activity.
Measurements of the GS activity in the presence of
various amounts of phosphinothricin revealed the same
degree of enzyme inhibition by PPT in both cell types,
suggesting that no structural change of the GS-protein
had occured during the selection of the resistant
variant.

Messenger RNA was isolated from wild type and
resistant cells using a guanidinium isothiocyanate
RNA-extraction method, followed by the separation of
the mRNA through an oligo dT-cellulose column. 4
microgram mRNA could be isolated per gram of cell
material. In vitro translation of the isolated mRNA
from both cell lines yielded patterns of polypeptides
similar to the in vivo pattern obtained in ^{35}S-methio-
nine labelled extracts. This indicated that the
isolated mRNA was fairly undegraded. While the in
vivo labelled proteins showed a striking difference in
the amount of the 40-42 KD polypeptide, the differ-
ence between wild type and variant cell line in the in
vitro translated protein patterns was not pronounced.

First and second strand cDNA were synthesized from
the mRNA population of the PPT-resistant variant.
After S_1-cleavage and poly C-tailing of the cDNA, the
DNA was annealed to poly G-tailed pBR 322 vector DNA.
E. coli strain MC1061 was transformed with the recir-
cularized vector.

3.5x10^4 colonies were obtained per mg of annealed vector DNA. 80% of the colonies were ampicillin (amp) sensitive, and 50% of the amp sensitive clones had inserts which could be detected by Pst digestion of the plasmids. 10% of the inserts were longer that 1,000 base pairs.

1,800 colonies were grown on nitrocellulose filters and the filters were probed with ^{32}P-labelled first strand cDNA probe having increased percentage of GS-specific sequences. Amongst such clones it was expected to find a GS-specific cDNA clone. A GS-cDNA clone from a _Phaseolus_ cDNA library prepared from mRNA of root nodules was obtained from an outside source. This GS-cDNA clone enabled the identification of one alfalfa cDNA clone in the cDNA library, which hybridized strongly to the _Phaseolus_ GS-DNA.

The insert DNA of the alfalfa GS-cDNA clone was sequenced. The alfalfa and the _Phaseolus_ GS-cDNAs were then used as probes to characterize the difference between the wild alfalfa cells and the variant, GS-overproducing cells.

Total genomic DNA was prepared from alfalfa leaves, from wild-type cell line and from three sublines of the resistant cell line which had been selected in presence of 6x10^{-4}M, 2x10^{-3}M and 3x10^{-3}M L-phosphinothricin. The DNA was digested with Bam HI, Eco RI, and HindIII, and a combination of 2 enzymes. After agarose gel electrophoresis of the digested DNAs, the DNA fragments were transferred onto nitrocellulose filters by Southern blotting. The filter-bound DNA was hybridized with ^{32}P-labelled c-DNA

inserts of the alfalfa and the Phaseolus GS-clones. DNA of the same size hybridized with both probes. One predominant band hybridizes with the same intensity in all 5 DNA samples with the alfalfa probe. A second band, which is barely visible in the wild type DNA digest, strongly hybridizes with DNA of the resistant cell lines. The highest degree of hybridization was observed with DNA of the highly resistant cell lines, indicating amplification of a DNA fragment during the development of the resistance. The alfalfa cDNA probe strongly hybridized to the amplified DNA fragment and, to a lesser extent, to the non-amplified DNA with GS homology.

In order to confirm the reason why the variant alfalfa cell line had become resistant to PPT, both Northern and Southern blots were repeated using wild type and variant cell lines. The Northern blot indicated that there is an increase of about 8 fold in glutamine synthetase mRNA from wild type. When Southern blots were done using genomic DNA from both cell lines, there was a clear indication of gene duplication of glutamine synthetase in the mutant alfalfa cell line. The duplication seems to be 5 to 15 fold above the unduplicated glutamine synthetase gene as estimated by hybridization. It also appeared to be an exact duplication, that is, only one band increased in hybridization by 7 to 11 fold.

Example 2

Isolation and Characterization
of Glutamine Synthetase
from the PPT-Resistant Cell Culture.

Materials and Methods

CNBr Cleavage

Glutamine synthetase (12 nmol) was dissolved in 1 mL of 70% formic acid, 5 mg CNBr was added, and the cleavage proceeded for 24 hr at room temperature (Gross, Meth. Enzymol., 11:238-255 (1967)). After dilution with distilled water (6 mL), the mixture was lyophilized twice.

High Performance Liquid Chromatography of CNBr Peptides

Reverse-phase high performance liquid chromatography (HPLC) was carried out on an Ultrapore RPSC (0.46 ID x 7.5 cm) at 40°C using 0.1% trifluoroacetic acid (TFA) (Bennett et al., Biochem. J., 168:9-13 (1977)) and a linear gradient of 0-60% acetonitrile (30 min; flow rate, 0.5 mL/min). The CNBr peptides were dissolved in the minimum volume of 6 M guanidine hydrochloride in 0.1% TFA. Chromatographic peaks from several separations were collected manually, pooled, and lyophilized. Detection was at 214 nm using a Beckman 160 detector.

Amino acid analysis

Amino acid compositions were determined after acid hydrolysis of samples in sealed, evacuated

-32-

tubes (Pyrex®, culture, rimless, 6x50 mm) at 110°C for 24 hr in constant boiling HCl (0.025 mL) (Moore, Chemistry and Biology of Peptides, Ann Arbor Science, Ann Arbor, Michigan, 629-653 (1972)). Using a Beckman 6300 amino acid analyzer equipped with two Hewlett-Packard 3390A integrators, ninhydrin and two channel (440 and 570 nm) integration provided analysis of all amino acids, except tryptophan, cysteine, asparagine, and glutamine, with confidence values of 1-7% at 100 pmol/amino acid, and with a lower limit of detection of 25 pmol. Analyses were done 3-5 times on each sample and background controls.

Protein sequence analysis:

Automated Edman degradation was performed with an Applied Biosystems 470A sequencer. The sequence program was developed earlier for the gas-liquid solid phase peptide and protein sequencer (Hewick et al., J. Biol. Chem., 256: 7990-7997 (1981)). The program contains one coupling step (44°C, 26 min) and a single cleavage step (44°C, 6.7 min). Automated conversion of the 2-anilino-5-thiazolinone derivatives (Pth-amino acids) uses 25% trifluoroacetic acid (50 C, 33 min). Polybrene® (1.5 mg) (Tarr et al., Anal. Biochem., 84:622- 627 (1978); Klapper et al., ibid, 85:126-131 (1978)) was added to the glass filter disc in the cartridge prior to degradation of protein or peptides, and five sequence cycles were run to reduce contaminants derived from

-33-

Polybrene®. Angiotensin II (1 nmol) was added to the cartridge filter, and ten degradative cycles were completed. Sequencing of angiotensin allowed an assessment of the chemical and mechanical operation of the sequencer prior to the sequencing of an unknown. Protein sequence analysis of sperm whale apomyoglobin (100-200 pmol) routinely demonstrated an initiated yield of 45-55%, average repetitive yield of 92-93%, and an average lag per cycle of 2-3%. All Pth-amino acids were identified by reverse-phase high performance liquid chromatography on a cyano column (0.45 x 25 cm) using a 15 mM sodium acetate buffer (pH 5.5), and a complex gradient of acetonitrile/methanol (92.5:7.5, v/v), based on a system developed by Hunkapiller and Hood, Methods Enzymol., 91:486-493 (1983). This HPLC system separated the Pth-amino acids and internal standard (methyl ester of Pth-Glu) from the major contaminants: a dithiothreitol-adduct, N-dimethyl-N'-phenylthiourea, and diphenylthiourea. Methionine and proline were not routinely separated, but a modification of the gradient separated these two Pth-amino acids. Internal standard (200 pmol) was added to each cycle collection, and the samples were dried in a Speedvac Concentrator with an RH100-6 rotor. The dried samples were dissolved in 0.025 mL of water/acetonitrile (80:20, v/v), and an aliquot (0.017 mL)(68% of total sample) injected automatically. The Pth-amino acids were detected by UV absorbance at 254 nm using a Beckman 160 detector.

Purification Scheme

The process of purification is briefly summarized in the following scheme:

100 gm. frozen cells

Add 50 mM Tris-pH 7.5

Use blender low speed (30 seconds)

High speed (2 minutes)

Spin 8K rpm, 5°, 10 minutes

Supernatant   7.5% v/v 1% Protamine Sulfate,

15 minutes, 4°

Spin 8K rpm, 5°, 10 minutes

Supernatant   1.5% v/v 1M $MgSO_4$

pH to 6.8 with 1N acetic acid

Me to 7 mM

Bind to H.A., 4°, 15 minutes

Wash H.A. 5X with 50 mM Tris pH 7.5

0.28M $MgSO_4$

10% ethylene glycol

Elute GS 5X with 50 mM Tris pH 7.5

0.5M $MgSO_4$

16.7% ethylene glycol

↓

Pool eluted fractions, dialyze against 10 mM Tris

pH 6.8

10 mM Mg

$MgSO_4$

↓

G-100 column, pool activity peak

100 grams of frozen variant alfalfa tissue culture cells were mixed with dry ice and ground to a fine powder in a blender. After thawing, the mixture was spun at 8,000 rpm at 4° for 20 minutes. Protamine sulfate precipitation was carried out on the supernatant to remove nucleic acid. The $MgSO_4$ concentration was brought to 10 mM, the BMe to 7 mM, and the pH adjusted to 6.8 with 1N acetic acid. The crude exact was bound to H.A. The H.A. was washed batchwise 5 times with 10 mM Tris, pH 7.5, 0.28M $MgSO_4$, and 10% ethylene glycol. Glutamine synthetase was eluted by bringing the $MgSO_4$ concentration to 0.5M. Five batch elutions were done. After dialyzing against 10 mM Tris, pH 7.5, 10 mM $MgSO_4$, it was loaded onto a G-200 column. Fractions with good glutamine synthetase activity were pooled. By SDS acrylamide gel and silver staining, the protein was better than 95% pure. An amino acid composition showed the composition of alfalfa glutamine synthetase to be nearly the same as that of a published composition from soy bean (Table 1)

-36

Table 1

Amino Acid Composition of Alfalfa Glutamine Synthetase

Amino Acid	Soy Bean* Root (mole%)	Pea Leaf (mole %)	Alfalfa (mole % + C.V.)
Ala	8.4	6.9	8.7 (+0.4)
Arg	5.6	4.0	5.0 (+0.5)
Asx	10.4	10.7	11.4 (+0.6)
Cys	0.8	N.D	N.D
Glx	10.0	10.1	9.9 (+0.5)
Gly	10.6	19.1	13.5 (+0.7)
His	2.2	3.6	3.1
Ile	6.6	5.6	8.4 (+0.4)
Leu	6.7	6.5	7.6 (+0.9)
Lys	5.9	5.7	6.8 (+0.3)
Met	1.6	N.D.	0.6 (+0.3)
Phe	2.8	2.6	2.3
Pro	6.1	8.8	8.0 (+0.6)
Ser	5.4	7.8	6.5 (+0.4)
Thr	5.1	4.9	5.6 (+0.3)
Trp	1.5	N.D.	N.D.
Tyr	3.9	0.5	3.4 (+0.7)
Val	6.3	4.8	4.4 (+1.5)

* R.H. McParland, et al., Biochem. J. (1976) 153:597-606
N.D. = None Deleted
C.V. = Confidence Value (S.D./mean)

<u>Sequencing</u>

A first attempt at sequencing was initiated using the purified native protein. There was no obtainable sequence, indicating that the NH_2- terminal was naturally blocked. A CNBr cleavage was carried out, and the fragments were separated by HPLC. The amino acid composition of the protein indicated that there were only two to five methionines. Two of the HPLC separated CNBr fragments yielded useful sequence information.

Sequencing of purified glutamine synthetase fragment was then carried out. The results are as follows:

Arg-Glu-Asp-Gly-Gly-Tyr-Glu-Val-Ile-
Leu-Lys-Ala-Ile-Glu-Lys-Leu-Gly-Lys-
Lys-(Glu/His)-Lys-Glu-His-Ile-Ala-
Ala-Tyr-Gly-Gly-Gly-Asn

This sequence corresponds to part of the sequence obtained from the complete variant alfalfa cDNA clone. The deduced protein sequence is shown on the following page.

```
1    ATG TCT CTC CTT TCA GAT CTT ATC AAC CTT GAC CTC TCC GAA ACC ACC GAG AAA ATC ATC GCC GAA TAC ATA TGG    75
     Met Ser Leu Leu Ser Asp Leu Ile Asn Leu Asp Leu Ser Glu Thr Thr Glu Lys Ile Ile Ala Glu Tyr Ile Trp

76   ATT GGT GGA TCT GGT TTG GAC TTG AGC AAA GCA AGG ACT CTA CCA GGA CCT GTT ACT GAT CCA TCA CAG CTT        150
     Ile Gly Gly Ser Gly Leu Asp Leu Ser Lys Ala Arg Thr Leu Pro Gly Pro Val Thr Asp Pro Ser Gln Leu

151  CCC AAG TGG AAC TAT GAT GGT TCA AGT ACA GGT CAA GCT CCA GGT GAA GAT TCC GAA GTA ATC ATC TAC CCA CAA    225
     Pro Lys Trp Asn Tyr Asp Gly Ser Ser Thr Gly Gln Ala Pro Gly Glu Asp Ser Glu Val Ile Ile Tyr Pro Gln

226  GCC ATT TTC AAG GAC CCA TTT AGA AGA GGT AAC AAT ATC TTG GTT ATG TGT GAT GCA TAC ACT CCA GCA GGT GAG    300
     Ala Ile Phe Lys Asp Pro Phe Arg Arg Gly Asn Asn Ile Leu Val Met Cys Asp Ala Tyr Thr Pro Ala Gly Glu

301  CCC ATT CCC ACC AAC AAG AGA CAT GCA GCT GCC AAG ATT TTC AGC CAT CCT GAT GTT GTT GCT GAA GTA CCA TGG    375
     Pro Ile Pro Thr Asn Lys Arg His Ala Ala Ala Lys Ile Phe Ser His Pro Asp Val Val Ala Glu Val Pro Trp

376  TAT GGT ATT GAG CAA GAA TAC ACC TTG TTG CAG AAA GAC ATC AAT TGG CCT CTT GGT TGG CCA GTT GGT GGT TTT    450
     Tyr Gly Ile Glu Gln Glu Tyr Thr Leu Leu Gln Lys Asp Ile Asn Trp Pro Leu Gly Trp Pro Val Gly Gly Phe

451  CCT GGA CCT CAG GGA CCA TAT TAT TGT GGC GTT GGT GCA GAT AAG GCT TTT GGA AGA GAT ATT GTT GAC TCA CAT    525
     Pro Gly Pro Gln Gly Pro Tyr Tyr Cys Gly Val Gly Ala Asp Lys Ala Phe Gly Arg Asp Ile Val Asp Ser His

526  TAC AAA GCC TGT CTT TAT GCC GGC ATC AAC ATC AGT GGA ATC AAT GGT GAA GTG ATG CCT GGT CAA TGG GAA TTC    600
     Tyr Lys Ala Cys Leu Tyr Ala Gly Ile Asn Ile Ser Gly Ile Asn Gly Glu Val Met Pro Gly Gln Trp Glu Phe

601  CAA GTT GGT CCC TCA GTT GGT ATC TCT GCT GGA GAT GAG ATA TGG GTT GCT AGG TAC ATT TTG GAG AGG ATC ACT    675
     Gln Val Gly Pro Ser Val Gly Ile Ser Ala Gly Asp Glu Ile Trp Val Ala Arg Tyr Ile Leu Glu Arg Ile Thr

676  GAG GTT GCT GGT GTG GTG CTT TCC TTT GAC CCA AAA CCA ATT CCA GGT GAT TGG AAT GGT GCA GGT GCT CAC ACA    750
     Glu Val Ala Gly Val Val Leu Ser Phe Asp Pro Lys Pro Ile Pro Gly Asp Trp Asn Gly Ala Gly Ala His Thr

751  AAT TAC AGC ACC AAG TCT ATG AGA GAA GAT GGT GGC TAT GAA GTC ATC TTG AAA GCA ATT GAG AAG CTT GGG AAG    825
     Asn Tyr Ser Thr Lys Ser Met Arg Glu Asp Gly Gly Tyr Glu Val Ile Leu Lys Ala Ile Glu Lys Leu Gly Lys

826  AAG CAC AAG GAG CAC ATT GCT GCT TAT GGA GAA GGG AAC GAG CGT AGA TTG ACA GGG CAT CAT GAG ACA GCT GAC    900
     Lys His Lys Glu His Ile Ala Ala Tyr Gly Glu Gly Asn Glu Arg Arg Leu Thr Gly His His Glu Thr Ala Asp

901  ATT AAC ACC TTC TTA TGG GGT GTT GCA AAC CGT GGT GCG TCG ATT AGA GTT GGA AGG GAC ACA GAG AAA GCA GGG    975
     Ile Asn Thr Phe Leu Trp Gly Val Ala Asn Arg Gly Ala Ser Ile Arg Val Gly Arg Asp Thr Glu Lys Ala Gly

976  AAA GGT TAT TTC GAG GAT AGG AGG CCA TCT AGC AAC ATG GAT CCA TAT GTT GTT ACT TCC ATG ATT GCA GAC ACC    1050
     Lys Gly Tyr Phe Glu Asp Arg Arg Pro Ser Ser Asn Met Asp Pro Tyr Val Val Thr Ser Met Ile Ala Asp Thr

1051 ACC ATT CTC TGG AAA CCA TAA   1071
     Thr Ile Leu Trp Lys Pro End
```

-39-

Preparation of a Functional Genomic
Sequence Coding for Glutamine Synthetase

Total genomic DNA was obtained from the alfalfa tissue culture using a protocol similar to that used to obtain that of Phaseolus root nodules (Cullimore, J.D., and Miflin, B.J., FEBS Letters, 158:107-112 (1983)), and digested totally with Bam HI. The material was then size fractionated in a potassium acetate 5-20% gradient and separated into aliquots. Each aliquot was then run on a 1% agarose gel and probed in a Southern transfer system with cDNA glutamine synthetase probe obtained from a Pst cut of the coding sequence probe shown above. Positive hybridizations were then selected for fragments of size greater than 4 Kb. These were ligated to a BV-2 lambda vector, 100,000 phages were plated and probed with the glutamine synthetase cDNA probe as above. Four positive plaques were obtained. These were then grown and plaque-hybridization purified for 3-4 rounds. A purified 4 Kb genomic clone was isolated and religated into Ml3mp9. This clone was sequenced from both ends. In addition, the clone was also fragmented with Hae III, subcloned into MP-9 and again sequenced.

In this manner, this 4 Kb 5' fragment (as well as an 8 Kb 3' fragment also obtained from the digestion of the genomic DNA) can be completely sequenced. Both of these fragments together will provide a complete functional gene, the reading frame being predicted from the protein sequence information at hand, the known GS molecular weight and the relative incidence of stop triplets in the three possible reading frames.

With a complete functional sequence for GS at hand, the same can be expressed by any of the methods described previously.

Having now fully described this invention it will be understood that the same can be operated within a broad and equivalent range of structures, products, processes, and uses without effecting the spirit or scope of the invention or any embodiment thereof.

CLAIMS

1.   A plant cell which is resistant to a herbicidal glutamine synthetase (GS) inhibitor, wherein said resistance is caused by plant cell levels of GS activity which, when present in an otherwise herbicidal GS-inhibitor sensitive plant cell, render said cell substantially resistant to said herbicidal GS-inhibitor.

2.   The resistant plant cell of Claim 1 wherein said levels of GS activity are present by virtue of said plant cell having a significantly larger number of copies of the wild GS gene therein, than the corresponding sensitive plant cell.

3.   The plant cell of Claim 2 wherein the number of copies of the wild GS gene in said plant cell is such that the levels of GS activity produced therefrom render said cell substantially resistant to said herbicidal GS-inhibitor.

4.   The plant cell of any of Claims 1 or 2 wherein the wild gene for GS in said plant cell is substantially amplified.

5.   The plant cell of Claim 1 carrying a genetic sequence combination which comprises:

(a) a first genetic sequence coding for glutamine synthetase (GS) functional in said plant cell, operably linked to

(b) a second genetic sequence capable of increasing the levels of expression of said first genetic sequence such that when such combination is present in an otherwise herbicidal GS-inhibitor sensitive plant cell, said cell is substantially resistant to said herbicidal GS-inhibitor.

6.   The plant cell of Claim 5 wherein said combination is integrated in the genome of said plant cell.

7.   The plant cell of Claim 5 wherein said combination is present in a Ti plasmid of <u>Agrobacterium tumefaciens</u>.

8.   The plant cell of any of Claims 1, 2, 3, 5, 6 or 7 wherein said herbicidal GS-inhibitor is PPT.

9.   A transformed herbicidal GS-inhibitor resistant plant cell which, in its untransformed state, is herbicidal GS-inhibitor sensitive.

10.   The plant cell of Claim 9 wherein said herbicidal GS-inhibitor is PPT.

11.   A genetic sequence combination operable in a given plant cell which comprises:

(a) a first genetic sequence coding for glutamine synthetase functional in said plant cell, operably linked to

(b) a second genetic sequence capable of increasing the levels of expression of said first sequence such that when said combination is present in an otherwise herbicidal GS-inhibitor sensitive plant cell, said cell is substantially resistant to said herbicidal GS-inhibitor.

12. The genetic sequence combination of Claim 11 wherein said second genetic sequence is a promoter.

13. The genetic sequence combination of any of Claims 11 or 12 which is integrated in the genome of said plant cell.

14. The genetic sequence combination of any of Claims 11 or 12 which is present in the Ti plasmid of <u>Agrobacterium</u> <u>tumefaciens</u>.

15. The genetic sequence combination of any of Claims 11 or 12 wherein said herbicidal GS-inhibitor is PPT.

16. A herbicidal GS-inhibitor resistant plant comprising a cell according to any of Claims 1, 2, 3, 5, 6 or 7.

17.   The plant of Claim 16 wherein said herbicidal GS-inhibitor is PPT.

18.   A plant comprising the cell of Claim 9.

19.   A method of conferring herbicidal GS-inhibitor resistance to an otherwise herbicidal GS-inhibitor sensitive plant cell which comprises increasing the copies of the wild GS gene present in said sensitive plant cell.

20.   The method of Claim 19 wherein said herbicidal GS-inhibitor resistance is PPT resistance.

21.   A method of conferring herbicidal GS-inhibitor resistance to a plant cell which comprises transforming said cell with the genetic sequence combination of Claim 11.

22.   The method of Claim 21 wherein said herbicidal GS-inhibitor is PPT.

23.   A method of selectively controlling herbicidal GS-inhibitor sensitive plants which comprises contacting a herbicidal GS-inhibitor sensitive plant with plant controlling amounts of the herbicide GS-inhibitor, wherein said contact is carried out while simultaneously contacting a herbicidal GS-inhibitor resistant plant as claimed in Claim 16.

24. The method of Claim 23 wherein said herbicidal GS-inhibitor is PPT.

25. A recombinant DNA molecule comprising a genetic sequence coding for glutamine synthetase.

26. The molecule of Claim 24 which is a vehicle capable of transforming a plant cell.

27. A recombinant DNA (rDNA) molecule comprising a genetic sequence coding for glutamine synthetase, wherein said glutamine synthetase comprises the following polypeptide formula:
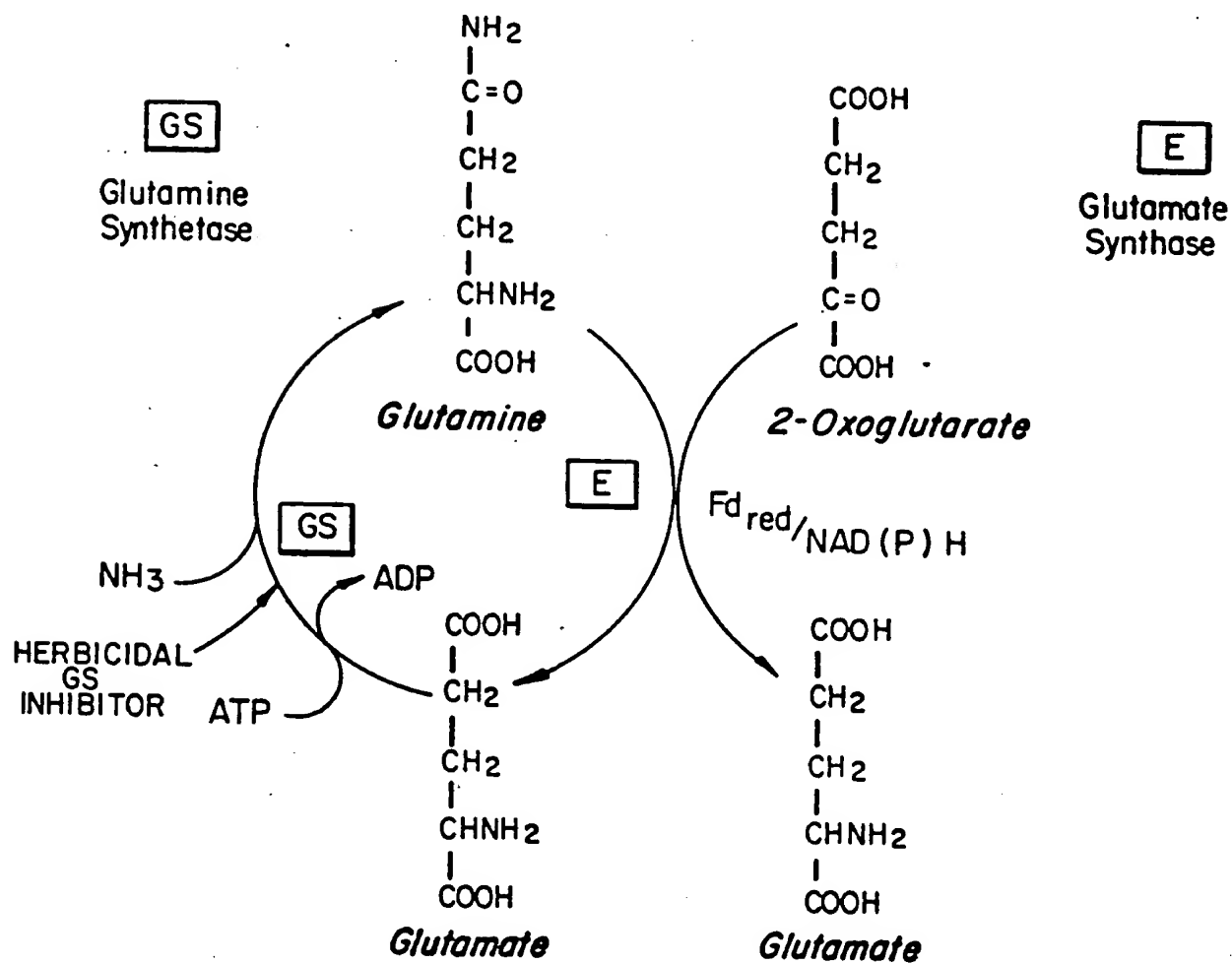
Met-Ser-Leu-Leu-Ser-Asp-Leu-Ile-Asn-
Leu-Asp-Leu-Ser-Glu-Thr-Thr-Glu-Lys-
Ile-Ile-Ala-Glu-Tyr-Ile-Trp-Ile-Gly-
Gly-Ser-Gly-Leu-Asp-Leu-Arg-Ser-Lys-
Ala-Arg-Thr-Leu-Pro-Gly-Pro-Val-Thr-
Asp-Pro-Ser-Gln-Leu-Pro-Lys-Trp-Asn-
Tyr-Asp-Gly-Ser-Ser-Thr-Gly-Gln-Ala-
Pro-Gly-Glu-Asp-Ser-Glu-Val-Ile-Ile-
Tyr-Pro-Gln-Ala-Ile-Phe-Lys-Asp-Pro-
Phe-Arg-Arg-Gly-Asn-Asn-Ile-Leu-Val-
Met-Cys-Asp-Ala-Tyr-Thr-Pro-Ala-Gly-
Glu-Pro-Ile-Pro-Thr-Asn-Lys-Arg-His-
Ala-Ala-Ala-Lys-Ile-Phe-Ser-His-Pro-
Asp-Val-Val-Ala-Glu-Val-Pro-Trp-Tyr-
Gly-Ile-Glu-Gln-Glu-Tyr-Thr-Leu-Leu-
Gln-Lys-Asp-Ile-Asn-Trp-Pro-Leu-Gly-
Trp-Pro-Val-Gly-Gly-Phe-Pro-Gly-Pro-
Gln-Gly-Pro-Tyr-Tyr-Cys-Gly-Ala-Gly-

```
Ala-Asp-Lys-Ala-Phe-Gly-Arg-Asp-Ile-
Val-Asp-Ser-His-Tyr-Lys-Ala-Cys-Leu-
Tyr-Ala-Gly-Ile-Asn-Ile-Ser-Gly-Ile-
Asn-Gly-Glu-Val-Met-Pro-Gly-Gln-Trp-
Glu-Phe-Gln-Val-Gly-Pro-Ser-Val-Gly-
Ile-Ser-Ala-Gly-Asp-Glu-Ile-Trp-Val-
Ala-Arg-Tyr-Ile-Leu-Glu-Arg-Ile-Thr-
Glu-Val-Ala-Gly-Val-Val-Leu-Ser-Phe-
Asp-Pro-Lys-Pro-Ile-Lys-Gly-Asp-Trp-
Asn-Gly-Ala-Gly-Ala-His-Thr-Asn-Tyr-
Ser-Thr-Lys-Ser-Met-Arg-Glu-Asp-Gly-
Gly-Tyr-Glu-Val-Ile-Leu-Lys-Ala-Ile-
Glu-Lys-Leu-Gly-Lys-Lys-His-Lys-Glu-
His-Ile-Ala-Ala-Tyr-Gly-Glu-Gly-Asn-
Glu-Arg-Arg-Leu-Thr-Gly-Arg-His-Glu-
Thr-Ala-Asp-Ile-Asn-Thr-Phe-Leu-Trp-
Gly-Val-Ala-Asn-Arg-Gly-Ala-Ser-Ile-
Arg-Val-Gly-Arg-Asp-Thr-Glu-Lys-Ala-
Gly-Lys-Gly-Tyr-Phe-Glu-Asp-Arg-Arg-
Pro-Ser-Ser-Asn-Met-Asp-Pro-Tyr-Val-
Val-Thr-Ser-Met-Ile-Ala-Asp-Thr-Thr-
Ile-Leu-Trp-Lys-Pro
```
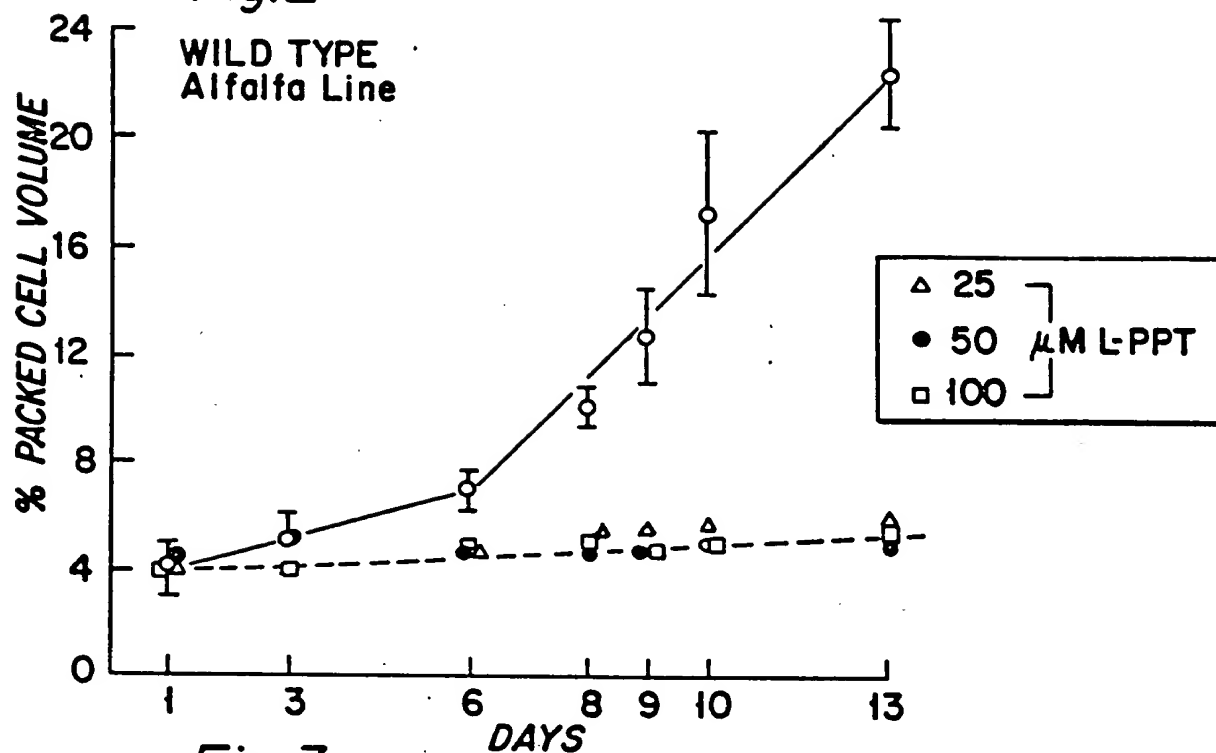
Fig. 1

THE GLUTAMATE SYNTHASE CYCLE

Fig.2
WILD TYPE
Alfalfa Line

△ 25 ⌐
● 50 μM L-PPT
□ 100 ⌐

Fig.3
VARIANT (L-PPT^R)
Alfalfa Line

△ 100 ⌐
● 200 μM L-PPT
□ 500 ⌐